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Molecular Identification of *Trichoderma* spp. in Garlic and Onion Fields and *In Vitro* Antagonism Trials on *Sclerotium cepivorum*

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ABSTRACT: *Trichoderma* species are non-pathogenic microorganisms that protect against fungal diseases and contribute to increased crop yields. However, not all *Trichoderma* species have the same effects on crop or a pathogen, whereby the characterization and identification of strains at the species level is the first step in the use of a microorganism. The aim of this study was the identification – at species level – of five strains of *Trichoderma* isolated from soil samples obtained from garlic and onion fields located in Costa Rica, through the analysis of the ITS1, 5.8S, and ITS2 ribosomal RNA regions; as well as the determination of their individual antagonistic ability over *S. cepivorum* Berkeley. In order to distinguish the strains, the amplified products were analyzed using MEGA v6.0 software, calculating the genetic distances through the Tamura-Nei model and building the phylogenetic tree using the Maximum Likelihood method. We established that the evaluated strains belonged to the species *T. harzianum* and *T. asperellum*; however it was not possible to identify one of the analyzed strains based on the species criterion. To evaluate their antagonistic ability, the dual culture technique, Bell's scale, and the percentage inhibition of radial growth (PIRG) were used, evidencing that one of the *T. asperellum* isolates presented the best yields under standard, solid fermentation conditions.

Keywords: ribosomal RNA, antagonistic test, phylogenetic analysis.

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INTRODUCTION

Species from the genus *Trichoderma* include a vast number of strains with economic importance based on their biological control potential, being commercialized as biopesticides, biofertilizers, and soil enhancers. They are non-pathogenic microorganisms that provide protection against fungal diseases caused by *Phytophthora*, *Rhizoctonia*, *Sclerotium*, *Pythium*, and *Fusarium* genera (Peteira et al., 2001); additionally, they promote high yields in crops (Ezziyyani et al., 2004a). These traits derive from their ability to produce antifungal metabolites, release hydrolytic enzymes, and their mycoparasitic behavior, as well as the production of other substances that enhance plant growth (Suárez-Mesa et al., 2008).

In garlic (*Allium sativum*) crops, positive results of biological control against the pathogen *Sclerotium cepivorum* – responsible for white rot – have been observed through the use of native isolates of *Trichoderma* obtained from the region of Llano Grande, in Cartago, Costa Rica (Astorga Quirós et al., 2014a). However, not all *Trichoderma* species have the same effects over a pathogen or a specific crop. Hence the strain characterization and identification at a species level is the first step required to select the correct microorganism, since it determines which isolate presents a higher antagonistic effect over another particular organism (Rubio et al., 2005).

Studies on *Trichoderma* diversity and speciation have determined that the ribosomal RNA regions ITS1, 5.8S, and ITS2 are among the most informative sequences, in taxonomical and phylogenetic terms (Guigón-López et al., 2010).

The hypotheses was that *Trichoderma* is a genus present in the onion and garlic's rhizosphere in soil in Costa Rica, and the isolates obtained had different levels of antagonistic activity against *S. cepivorum*. The objective of this study was the identification, at a species level, of five different strains of *Trichoderma* isolated from soil samples gathered at garlic (*Allium sativum*) and onion (*Allium cepa*) production fields from the region of Llano Grande in Cartago, where microorganisms had not been implemented, through the study of the ribosomal RNA (rRNA) regions ITS1, 5.8S and ITS2; as well as the *in vitro* determination of the antagonistic effect of each strain on *Sclerotium cepivorum* Berkeley.

MATERIAL AND METHODS

Total DNA extraction and amplification of ITS1, 5.8S and ITS2 rRNA regions

Five strains of *Trichoderma* spp., previously purified and identified as A, B, C, B₄ and D₄, were cultured in Petri dishes with Potato Dextrose Agar (PDA) medium, acidified with lactic acid. These strains were maintained at room temperature during three days, in order to allow growth of sufficient mycelium to perform total DNA extraction.

The DNA extractions were achieved through the use of Wizard Genomic DNA Purification Kit for A, B₄ and D₄ strains, while for B and C strains, the DNeasy® Plant Handbook kit was used. In both cases, the extraction was performed as described per the manufacturer's specifications, with a modification in the initial stage, with samples being exposed to liquid nitrogen in lysis buffer for 2 min, for the latter disruption of the material using a Retsch® macerator. DNA concentration and purity were quantified through spectrophotometry, using 260 and 280 nm absorbance values.

The amplification of the ITS1, 5.8S and ITS2 regions generated from the total DNA extractions was developed according to the method proposed by Guigón-López et al. (2010), using SR6R (5'-AAGTAGAAGTCGTAACAAGG-3') and LR1 (5'-GGTTGGTTTCTTTTCCT-3') oligos. The thermocycling program used was: 1 cycle at 94 °C for 2 min, 30 cycles at 94 °C for 30 s, 30 cycles at 50 °C cycles for 45 s, 30 cycles at 72 °C for 1 min and a final 72 °C cycle for 15 min. The PCR products were run using 1 % agarose gel during 30 min. The MassRuler™

DNA Ladder Mix molecular marker was used. Sequencing of the amplified products was performed sending 20 µL of each sample to the MacroGen Inc company (USA).

Sequence editing and alignment for phylogenetic reconstruction

The sequences were first edited with the BioEdit software (Hall, 1999), then assembled through the CAP3 tool (Huang and Madan, 1999); finally they were aligned to the homologous sequences through the tool MUSCLE, from EMBL-EBI (Edgar, 2004). The multiple alignment file was examined with the MEGA v6.0 software (Tamura et al., 2013) in order to calculate the genetic distance between the strains based on the number of base pair substitutions between the sequences. The positions with missing data were eliminated using the Tamura-Nei model. The phylogenetic trees were built through the Maximum Likelihood (ML) method, with a 500 repetition bootstrap.

Antagonistic activity of the isolated *Trichoderma* strains over *S. cepivorum*

The dual culture technique was used as described by Astorga Quirós et al. (2014b). For this study, five repetitions were performed for each strain, which were incubated under the same conditions for a 10-days period.

The antagonistic effect of the *Trichoderma* isolates was verified according to the scale proposed by Bell et al. (1982). Additionally, as a second indicator to test their antagonistic activity, the percentage inhibition of radial growth (PIRG) was determined employing the formula presented by Ezziyyani et al. (2004b), $PIRG = (R_1 - R_2)/R_1 \times 100$, where R_1 corresponds to the pathogen's growth radius (control) and R_2 is the growth radius of the pathogen once it is inoculated with *Trichoderma*. In order to establish the value for R_1 , five Petri dishes with PDA were inoculated with a disk 5 mm in diameter with 10-days of age active mycelium of *S. cepivorum*, which allowed us to determine the average radius of these cultures. A previous assay had been performed to corroborate the feasibility of reproducing these microorganisms in solid state fermentation. Considering these three characteristics (viz. Bell's scale, PIRG and reproduction feasibility), the potential application of each isolate, to be used in *S. cepivorum* biological control programs, was evaluated.

Aside from the previously mentioned factors, the ease of reproducing the different stains under a standardized protocol for solid state fermentation was also determined.

Reproduction Tests for every isolate

The ease of reproduction strains was assessed by the conidial concentration produced and viability of conidia. To do this, sterile trays with 500 g of hydrated rice were inoculated with 30 mL of a solution of spores at a concentration of 1.0×10^6 conidia/mL. Ten trays per strain were inoculated and incubated for 12 days at 28 °C, 80 % RH. Subsequently, for measuring the conidial concentration, serial dilutions in sterile distilled water were performed with 10 g of every tray. Counts in Neubauer's Chamber were performed using 10^4 dilution. The average of the samples was calculated for each strain. Viability was obtained by inoculating 0.2 mL of the same dilution on PDA medium. Ten plates were inoculated for each strain, and were incubated seven days at 28 °C. The colonies were counted and the average for each strain was obtained.

RESULTS

Total DNA extraction and amplification of ITS1, 5.8S and ITS2 rRNA regions

An average concentration of 39.21 µg/mL with an average A_{260}/A_{280} absorbance ratio of 1.74 of the total DNA from the five strains of *Trichoderma* was obtained. The amplification of the segment of interest was approximately 600 pb (Figure 1).

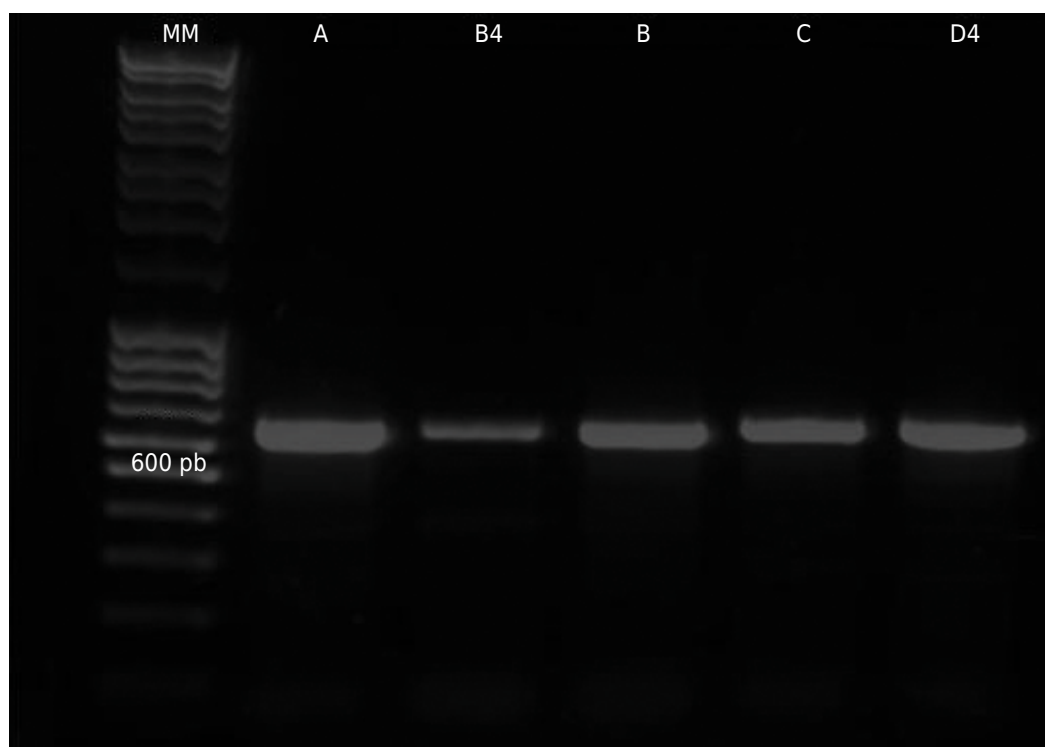


Figure 1. Electrophoretic bands generated during the optimized PCR reaction.

Sequence editing and alignment for phylogenetic reconstruction

The amplified sequences from each strain were compared to the GenBank database through the “Nucleotide Blast” search, where the A, B and C strains (GenBank: KM456214, KM456216 and KM456217, respectively) evidenced a 99 % similarity with the KF723005.1 *T. asperellum* accession number. The D₄ strain (GenBank: KM456218) presented a 99 % similarity with the accession number EU280079.1, for *T. harzianum*. Regarding the B₄ strain (GenBank: KM456215), the oligonucleotides used were only able to identify the strain at a genus level, since there was not a significant percentage of similarity among the *Trichoderma* species to which it was compared in the database.

The development of the phylogenetic tree through the Maximum Likelihood (ML) method evidenced the existing differences between taxa, and presented a similar behavior to that observed when the sequenced PCR products were compared to the GenBank database, where the A, B and C strains were grouped together, while the strains B₄ and D₄ were more phylogenetically distant, being separated from the previously mentioned group.

Antagonistic activity of the isolated *Trichoderma* strains over *S. cepivorum*

The isolates presented divergent behaviors with regard to their antagonistic effects. According to Bell’s scale, the strains ranged between classes 1 and 3; with B₄, C and A being most aggressive (in that order), while B and D₄ showed a poor antagonistic activity. On the other hand, by analyzing the PIRG determinations, it was established that B₄ and C presented the best results (>50 %), being similar to the tendency observed in Bell’s scale (Table 1).

Reproduction Tests for every isolate

Concentration measurements and viable conidia were different for each of the isolates (Table 2). Strain C had the highest production of conidia and as many viable conidia per ml of the tested solution. By contrast, B₄ and D₄ produced lower amounts of conidia and viable conidia.

Table 1. Assessment of the *Trichoderma* spp. isolates in relation to their antagonistic activity and their reproduction ability in standard conditions for a solid state fermentation

Strain	Bell's Scale		Inhibition of radial growth	Biological Control Potential
	Value	Antagonistic potential		
			%	
A	2	Good	48.92	3
B	3	Poor	48.38	4
C	2	Good	53.50	1
B4	1	Very good	61.08	2
D4	3	Poor	47.30	5

Table 2. Measurement of the reproduction capacity in solid substrate fermentation

Strain	Conidia/mL	Viable conidia/mL	Reproduction
A	7.5×10^8	7.0×10^8	Good
B	8.5×10^8	6.5×10^8	Good
C	1.2×10^9	1.0×10^9	Good
B4	3.2×10^7	2.7×10^7	Poor
D4	4.5×10^7	2.0×10^7	Poor

For the assessment of reproduction capacity, it was determined that viable conidia concentrations less than 1.0×10^8 were not suitable for controlled solid substrate fermentation processes; these values are indicative of poor reproducibility.

DISCUSSION

Total DNA extraction and amplification of the rRNA ITS1, 5.8S, and ITS2 regions

The total DNA extraction procedure was executed using mycelium, in order to obtain the largest amount of genetic material, since various workers have mentioned the difficulties encountered when extracting total DNA from spores (e.g. Cassago et al., 2002; Lurá et al., 2003). The application of liquid nitrogen and the maceration along with the lysis buffer allowed a more efficient disruption, which solubilized a larger amount of nucleic acids, and ensured the deactivation of DNA degrading enzymes (Rønsted et al., 2007). The selected method allowed an average purity value of 1.74, which benefited the subsequent amplification and sequencing process of the bands of interest (Glase, 1995).

The average 642 bp amplified region from ITS1, 5.8S, and ITS2 (Figure 1) presented a considerable similarity to that reported by Guigón-López et al. (2010), who obtained a 605 bp sequence using the same oligos for the identification of *T. asperellum* and *T. longibrachiatum*. In this context, it is possible to confirm the reports of Beiggi and Piercey (2007), as well as Besse (2014), who suggested the efficiency of this sequence for the determination of phylogenetic and evolutionary relationships, since it contained the highly conserved 5.8S rRNA region, which is surrounded by two highly variable regions (ITS1 and ITS2) that differ even between species (Deng et al., 2008).

Sequence editing and alignment for phylogenetic reconstruction

The use of Blast demonstrated that, through the amplified rRNA region of the studied strains, it was possible to determine homologies up to a 99 % similarity with species identified in the database. Strain B₄ was the only exception, where the identification

reached the genus level. This outcome resembles Martin and Rygiewicz's (2005) report, where they suggest that obtaining low percentage results for identity through a Blast search can be due to the absence of reference sequences in the database.

On the other hand, the phylogenetic analysis of the strains in this study presented a similar tendency to the results gathered through the initial Blast search (Figure 2). Strains A, B and C were grouped in one same node, while D₄ was the most distantly related taxon. B₄ is more closely related to B and C; according to Mendoza-Revilla (2012), when more common ancestors are shared by two taxa, they are most related to each other. This is evidenced through the genetic distance matrix of the *Trichoderma* strains (Table 3), where the divergence of the taxa A, B and C in regards to B₄ is 0.06, while the divergence with D₄ is 0.70.

These results match those gathered by Sanz et al. (2004) in a study where they indicated that, due to the existing polymorphisms in each taxon, the rRNA coding sequence contributed to the distinction between *Trichoderma* genotypes; allowing exploration of the genetic diversity, as well as establishing associations among genotypes.

Antagonistic activity of the isolated *Trichoderma* strains over *S. cepivorum*

Trichoderma's biocontrolling ability is related to its development of different mechanisms that allow it to compete directly for space and nutrients. In fungi, these mechanisms are associated with the production of antifungal metabolites, the secretion of hydrolytic enzymes, and their mycoparasitic ability, being behaviors that are expressed at different intensities depending on the strain used (Michel Aceves et al., 2013).

The biocontrolling ability is shown in table 1, indicate the different antagonistic behaviors found in the same species. From Bell's scale as well as the PIRG determination, it was shown that B₄, C and A strains, respectively, had the best antagonistic activity yields, even though strains A, B and C are the same species based on the phylogenetic study (Figure 1). A similar trend was found by Hoyos-Carvajal et al. (2008) when studying *T. harzianum* species, where it was concluded that each isolate could have a specific mycoparasitic activity. Therefore, it is not possible to generalize and indicate that a specific *Trichoderma* species can be classified as a good antagonistic fungi, because that characteristic is specific to each isolate.

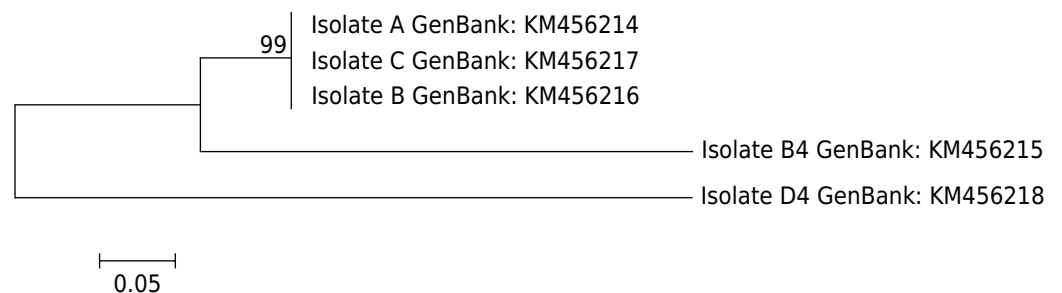


Figure 2. Phylogeny reconstruction based on the ITS1-5.8S-ITS2 sequences from the rRNA.

Table 3. Genetic distance matrix between the isolated strains of *Trichoderma*

Strain	A	B	C	D ₄	B ₄
A	-	-	-	-	-
B	0.01	-	-	-	-
C	0.01	0.01	-	-	-
D ₄	0.70	0.70	0.70	-	-
B ₄	0.06	0.06	0.06	0.71	-

Reproduction Tests for every isolate

The usefulness of these results in Costa Rican agriculture was carefully analyzed when the reproduction ability of each isolate in standard solid fermentation was studied. When performing the assay, as occurred in the antagonistic activity tests, differences between the same *Trichoderma* species were identified, with variations that depended on the isolate as well as biotic and abiotic factors. The fact that a strain had high values of conidial concentration and viable conidia in solid fermentation tests facilitates the transfer of results to farmers and companies producing microorganisms.

Therefore, considering these previously mentioned aspects, strain C – identified as *T. asperellum* – was selected as the most efficient isolate to be used in garlic crops to fight against the strain of *S. cepivorum* also isolated from the site of study.

CONCLUSIONS

Two different species of *Trichoderma* were identified As a result of the isolation of five fungal strains from a garlic agro-ecosystem; the strains were labeled A, B, and C being *T. asperellum*, and D₄ being *T. harzianum*.

Strain B₄ was not possible to identify at a species level, but this strain is more closely related to strains A, B and C than to D₄.

Some species had a good performance in the antagonistic activity test and can be considered as promising for biological control, but an isolate needs other desirable characteristics before being useful in the agroecosystem.

Strain labeled C presented the highest yield regarding antagonistic activity, considering its survival ability within a standard medium in a solid state fermentation. Therefore, this strain is the first option for being introduced into integrated management programs for controlling white rot in the region of Llano Grande in Costa Rica.

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